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Measurement of cromakalim-induced ^{87}Rb flux in intact cells by NMR

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^{87}Rb nuclear magnetic resonance signals from intracellular and extracellular Rb^+ can be distinguished by the use of shift reagents, thus permitting the simultaneous measurement of intracellular and extracellular Rb concentrations. When smooth muscle cells are suspended in a medium containing Rb^+ in place of K^+, there is a slow influx of Rb^+, which is greatly increased by the addition of cromakalim to the medium. The concentration of cromakalim required to stimulate influx is similar to that needed to stimulate ^{86}Rb efflux from isolated tissue.

Rubidium ion influx; NMR, ^{87}Rb-; Potassium channel; Cromakalim

1. INTRODUCTION

There has recently been considerable interest in the K^+ channel, since the demonstration that a number of antihypertensive agents, such as cromakalim (BRL 34915), act by opening K^+ channels (review [1]). Direct measurements of K^+ flux using $^{42}K^+$ are difficult, because of the short half-life of ^{42}K (12.4 h), and most measurements have been performed with ^{86}Rb, which seems to be a reasonable substitute for K in many cells [2–4]. We describe here an NMR-based method for measuring the flux of ^{87}Rb, which has advantages over ^{86}Rb measurements, in that it does not use radioactive nuclei, and it provides simultaneous direct measurement of intracellular and extracellular Rb concentrations with a time resolution of a few minutes [5]. It is based on the use of dysprosium(III) tripolyphosphate, Dy(PPP), to shift signals from extracellular Rb. This method has been applied successfully to distinguish intra- and extracellular Na en K [6–8] but has so far not been applied successfully to Rb, partly because of the large linewidth of the Rb signal and the small size of the Dy(PPP)-induced shift. The results obtained using this methodology are numerically similar to those obtained from ^{86}Rb measurements.

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2. MATERIALS AND METHODS

2.1. *Cell growth and harvesting*

The cells used in these experiments were from the established clonal line DDT_1 MF-2, derived from the ductus deferens of the Syrian hamster [9]. They have a number of properties characteristic of smooth muscle cells, including possessing receptors for α_1-adrenergic ligands [10] and leukotriene C_4 [11]. They were grown in 850-cm^2 roller bottles at 37 °C using Dulbecco's modified Eagle's medium (without sodium pyruvate) supplemented with 4.5 g/l glucose, 1% penicillin/streptomycin and 5% foetal calf serum (all from Gibco). Several reports suggest that the growth medium affects the expression of ion channels (eg [12]), and therefore to encourage differentiation once the cells had attained confluence, they were kept in medium containing 0.5% foetal calf serum for several days prior to harvesting. Preliminary experiments indicated that a period of 7–10 days in low serum concentrations was optimal, and this was used in all subsequent experiments. Harvesting was achieved without the use of trypsin simply by treatment with EDTA diamine and produced about 5×10^7 cells per roller bottle, of > 95% viability as judged by trypan blue exclusion.

2.2. *NMR experiments*

After harvesting, cells were kept in suspension in the growth medium until required (a maximum of 5 h). They were then spun down (2 min at 400 × g), washed with buffer, resus-

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pended, spun down and suspended in buffer at a concentration of about 5×10^7 cells/ml, after which they were placed in a 5 mm NMR tube and spun down to a pellet in a hand-operated centrifuge. The buffer was based on that described in [8], and contained 7 mM $DyCl_3$, 18.2 mM Na_5PPP, 10 mM Tris, 10 mM glucose, 54 mM NaCl (to give a total Na^+ concentration of 145 mM), and 5 mM RbCl (i.e. replacing all K^+ in the original buffer by Rb^+). It also contained 2 mM $CaCl_2$, and was made up in 10% D_2O on the day and was adjusted to a pH of 7.2.

^{87}Rb NMR spectra were collected at 37 °C on a Bruker AM-400 spectrometer operating at 130.93 MHz, using a 70 °C pulse width (approx. 25 μs), a spectral width of 42 kHz and an acquisition time of 12 ms. A series of spectra were collected for each sample, typically of 20 000 scans (4 min) each. Spectra were resolution-enhanced with a Lorentz-to-Gaussian transformation.

For each batch of cells, a control spectrum was acquired, after which the cells were resuspended, drugs were added, and the cells were spun down again. Acquisition of spectra started within 1 min of addition of drugs. NMR measurements were continued over a period of about 20 min. If no Rb transport was observed, the cells were resuspended and a further dose of cromakalim was added, after which NMR measurements were continued for another 20 min. Thereafter, cells retained >90% of their viability, but slowly lost viability over longer periods.

The cromakalim was prepared by Dr P.B. Kay (Roche).

3. RESULTS AND DISCUSSION

The suspension buffer caused a shift of 4 ppm in the extracellular ^{87}Rb resonance frequency, and no measurable shift in the intracellular signal, as compared to an external standard. This shift is smaller than the natural linewidth, so resolution enhancement was necessary to distinguish the two signals. In the absence of cromakalim, no intracellular signal could be observed initially, but over the course of several hours low levels of intracellular Rb^+ developed, indicating slow transport of Rb through the cell membrane. This effect was not sufficiently rapid to interfere with drug-induced measurements, but as a precaution, 20 μM ouabain was added to the cell suspension just before the addition of cromakalim, to inhibit Na^+K^+-ATPase, which has been shown to be the major transport system for Rb in a human atrial cell line [13].

On addition of cromakalim, intracellular Rb signals appeared rapidly, and Rb influx was complete within 10 min. Cromakalim solutions were made up in ethanol and diluted with buffer before adding to the NMR tube, so that the final ethanol concentration was 1% or less. Additions of ethanol/buffer mixtures alone had no effect on the NMR spectrum. A rigorous dose dependence relationship could not be established, because of the low sensitivity of the method, but it was possible to determine the minimum concentration required to observe Rb influx, which was 1.0 ± 0.4 μM. This is consistent with the minimum concentration needed to stimulate ^{86}Rb efflux in tissue preparations, observed by other authors [14,15], which are about 5-times those needed for electrical and mechanical effects.

Typical ^{87}Rb NMR spectra are shown in fig.1, in which the intracellular/extracellular signal intensity ratio after addition of cromakalim is approx. 1:3. In tightly packed cells, the ratio of intracellular/extracellular volume is only about 1:2, but the cell pellets formed here are not as tightly packed as this. We therefore estimate that the

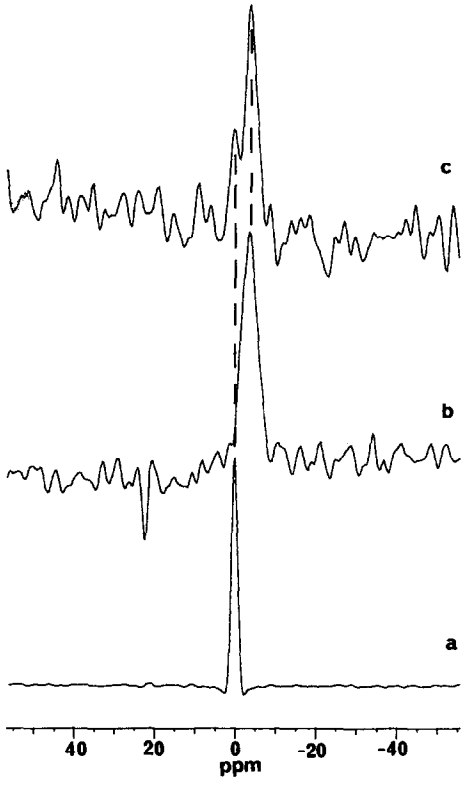

Fig.1. ^{87}Rb spectra, with resolution enhancement. (a) RbCl solution. (b) Cell suspension in buffer containing Dy(PPP). (c) As (b), after the addition of 1 μM chromakalim. Average over 4–15 min subsequent to addition of cromakalim. The dashed lines mark the resonance frequencies of intracellular and extracellular Rb, at 0 and -4 ppm, respectively.

typical intracellular Rb concentration after addition of cromakalim is only slightly greater than the extracellular concentration. However, if Rb and K are indistinguishable to the cell, the Nernst equation predicts that the intracellular Rb concentration at equilibrium should reach 10–30-times the extracellular level. The reason for the discrepancy is not yet clear.

These measurements are simple and rapid, as they do not need a pre-equilibration period in which the cells are loaded with Rb. They work in the opposite direction to the established methods using ^{86}Rb, which measure stimulated efflux, rather than influx. The fact that a smooth muscle cell line has been shown to undergo increased Rb influx at concentrations of cromakalim similar to those needed to stimulate Rb efflux in excised tissue opens up the possibility of a novel assay for the investigation of K^+ channel openers, and further confirms the role of K^+ channels in the pharmacological action of cromakalim.

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